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Prevalence of Precore Mutants in Anti-HBe-Positive Hepatitis B Virus Carriers in Germany

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Hepatitis B virus (HBV) precore mutants are associated often with highly productive infection in hepatitis B surface antigen (HBsAg) carriers lacking hepatitis B e antigen (HBeAg) but positive for anti-HBe, rendering serological identification of infectious individuals unreliable. Although considered initially to be limited mostly to the Mediterranean area, more recent studies suggest a significant presence of these mutants in northern European countries. The sequence of the precore region was determined and examined for mutations from HBV isolates of 99 German chronic HBsAg carriers positive for HBV-DNA and either HBeAg (n = 15) or anti-HBe (n = 84). In addition, clinical data of individuals carrying wild-type virus and those with precore mutants were compared. HBV precore mutants were found in more than half (44/84) of all HBeAg-negative, anti-HBe-positive virus carriers. There was no difference between carriers of wild-type and precore mutant HBV in the level of viremia or in the clinical course of chronic infection. In conclusion, HBV precore mutants are common in Germany and can therefore present a diagnostic problem for serological testing. However, precore mutants do not appear to have a detrimental effect on the course of chronic HBV infection. ***J. Med. Virol. 59:14–18, 1999.***

KEY WORDS: HBeAg; HBsAg; precore sequence

INTRODUCTION

Hepatitis B e antigen (HBeAg) is considered a marker for viral replication and infectivity in hepatitis B virus (HBV)-infected individuals, whereas the presence of anti-HBe is thought to indicate a low level or lack of viral production and often the absence of the virus in the blood. However, HBV variants have been described with mutations in the precore region that prevent HBeAg synthesis, despite continuing production of infectious virions [Carman et al., 1989; Lok et al., 1994]. The resulting diagnostic dilemma is emphasized by a recent report describing the transmission of HBV by anti-HBe-positive surgeons, all of whom were carriers of such precore mutants [The Incident Investigation Team et al., 1997]. Whereas initially these precore mutants were found mainly in individuals of Mediterranean origin [Carman et al., 1989; Brunetto et al., 1991], there is now good evidence that they are present in the northern part of Europe as well [Dienes et al., 1995; Tillmann et al., 1995]. The present study was carried out to investigate the prevalence of HBV precore mutants in the German population. To consider all changes leading to a loss of HBeAg and not only the most common mutation 1896 G→A (G to A transition at nucleotide 1896 introducing a stop in codon 28), the complete precore region of HBV isolates from HBV-DNA carriers was sequenced. Some researchers have described a higher incidence of fulminant hepatitis, more severe chronic courses, and poorer results from interferon treatment and liver transplantation in carriers of these mutations [Brunetto et al., 1991, 1993; Omata et al., 1991; Angus et al., 1995]. On the other hand, other researchers were not able to find an increased pathogenicity of the mutated virus [Okamoto et al., 1990; Tur Kaspa et al., 1992; Akarca et al., 1994; Lindh et al., 1996] Thus, due to the conflicting reports regarding clinical consequences of precore mutants, the possible impact of these mutations on the course of chronic infection was investigated.

MATERIALS AND METHODS

Patients

Sera from German patients with chronic HBV infection who were hepatitis B surface antigen (HBsAg)-positive and negative for antibodies to hepatitis C virus (HCV) and HIV (group A, n = 93) who were referred to the diagnostic laboratory from March 1992 to August

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TABLE I. HBeAg Status and HBV-DNA in 93 Unselected Germany Chronic HBsAg Carriers (Group A)

		HBV-DNA[a]	
HBeAg status	*n*	Positive	$\geq 10^5$ Copies/ml serum
HBeAg positive, anti-HBe negative	15	15 (100%)	13 (87%)
HBeAg negative, anti-HBe positive	78	58 (74%)	12 (15%)

HbeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.
[a]Detected by semiquantitative PCR (detection threshold 100 HBV genome copies/ml serum).

1997, were tested for HBeAg, anti-HBe, and HBV-DNA. In this patient group, serological testing had been initiated by the attending physician for the following reasons: follow-up evaluation of a known hepatitis B infection ($n = 43$); routine screening prior to surgical procedures ($n = 31$); diagnostic evaluation of hepatocellular carcinoma ($n = 4$); cirrhosis of the liver ($n = 2$); elevation of transaminases ($n = 1$); diagnostic evaluation of a dermatological disorder ($n = 5$); diagnostic evaluation of sepsis ($n = 3$); routine check-up prior to hemodialysis ($n = 1$); screening prior to hepatitis B vaccination ($n = 1$), or prior to blood donation ($n = 1$); and evaluation of a needle stick injury recipient ($n = 1$).

Also included in this study were sera sent for further investigation from 26 German patients known to have a chronic HBV infection and to be HBV-DNA-positive, HBeAg-negative, and anti-HBe-positive (group B), five of whom had been treated previously with interferon (in these five patients, only posttreatment sera were available).

HBV-DNA was isolated from all HBV-DNA-positive individuals, and the precore region was sequenced.

Detailed clinical data were available for all patients of group A and 23 patients of group B. Data were reviewed carefully to collect information about the patient history and clinical status. Information on suspected modes of infection or risk factors was available for 41 patients: previous blood transfusions ($n = 16$), travel to hyperendemic areas ($n = 14$), HBsAg-positive family members ($n = 6$), intravenous drug abuse ($n = 2$), living in an institution for the mentally handicapped ($n = 1$), tattooing ($n = 1$), and hemodialysis treatment ($n = 1$). Liver biopsies had been undertaken in 26 patients.

Serology

Serological diagnosis was carried out for HBsAg, HBeAg, anti-HBs, anti-HBc, anti-HBc-IgM, anti-HBe, anti-HCV, and anti-HIV with commercial microparticle enzyme immunoassays (AXSYM HBsAg™, AXSYM HBe™, AXSYM AUSAB™, AXSYM CORE™, AXSYM CORE-M™, AXSYM Anti-HBe™, AXSYM HCV™, AXSYM HIV-1/HIV-2™; Abbott Laboratories, Abbott Park, North Chicago, IL) according to the manufacturers' instructions.

Semiquantitative Determination of HBV-DNA

HBV-DNA was isolated from 200 μl serum using the QIAmp™ blood kit (Qiagen, Hilden, Germany). A 10-fold serial dilution of an HBV-DNA-containing plasmid ranging from 1 to 10^6 copies per test was amplified simultaneously with the patients' DNA. Polymerase chain reaction (PCR) was carried out using primers for the HBV surface and core regions as described previously [Weinberger et al., 1997]. All samples were tested in duplicate. After agarose gel electrophoresis, PCR products were blotted onto nylon membranes, hybridized with specific probes, and quantified densitometrically with a gel scanner using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1]decan}-4-yl)phenyl phosphate (CSPD, Serva, Heidelberg, Germany)-chemiluminescence as also described [Weinberger et al., 1997].

Sequencing of the Precore Region

The complete HBV precore region (codons 1–29, according to nucleotide positions 1814–1900) was analyzed: PCR was carried out with primers 1653 and 1972 [Yotsumoto et al., 1992] using the following concentrations: 250 μM of each dNTP, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3). Total volume was 100 μl with 2.5U Taq-polymerase. Reaction conditions were 5 min denaturation at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C. PCR products were purified with the QIAquick™-kit (Qiagen), and dye terminator cycle sequencing was carried out with an 373A ABI automated sequencer (PE Applied Biosystems Inc., Foster City, CA).

Statistical Analysis

The SPSS program package version 7.5 (SPSS Inc., Chicago, IL) was used to carry out Fisher's exact test (two-tailed), *t*-test (two-tailed), and Mann–Whitney *U*-test. The significance level was set at 5%. In addition, a log linear model was used to describe simultaneously the associations between the presence of a mutation, clinical sequelae, and age.

RESULTS

HBe-Status, HBV-DNA, and Precore Mutants in German Chronic HBsAg Carriers

Of 93 HBsAg-positive individuals in group A, all 15 HBeAg-positive and 58 (75%) of the 78 HBeAg-negative, anti-HBe-positive subjects were also positive for HBV-DNA by PCR (Table I). In two of the HBeAg-positive individuals, precore mutations introducing a stop into codon 28 were found (Table II). One patient was infected with the predominant precore stop mu-

TABLE II. HBV Precore Mutants in HBeAg-Positive and -Negative German HBsAg Carriers Positive for HBV-DNA

HBeAg-status	Patient group (*n*)	HBV-DNA Wildtype *n* (%)	HBV-DNA Mutant *n* (%)	Type of mutation (*n*)
HBeAg positive, anti-HBe negative	group A (15)	13 (87%)	2 (13%)	1896 G → A (1) 1897 G → A (1)[a]
HBeAg negative, anti-HBe positive	group A (58)	27 (47%)	31 (53%)	1896 G → A (21)[b] 1814 A → C (4)[b,c] 1816 G → T (2)[c] 1897 G → A (1) 1815 T → A (1) 1816 G → A (1) 1817 C → T (1)
	group B (26)	13 (50%)	13 (50%)	1896 G → A (8) 1897 G → A (2) 1814 A → T (2) 1814 A → C (1)

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.
[a]Wildtype sequence was also detectable.
[b]Wildtype sequence was also detectable in one carrier.
[c]One carrier of each group had a 1896 G → A mutation as well.

tant strain (1896 G→A). Another patient carried both wild-type and mutant HBV (1897 G→A).

Of the 58 HBeAg-negative and anti-HBe-positive individuals, 31 carried HBV with mutations known to prevent HBeAg synthesis: 22 by introducing a stop in codon 28 (1896 G→A, $n = 21$; 1897 G→A, $n = 1$), 1 by introducing a stop in codon 2 (1817 C→T), and 6 by abolishing the initiation codon (1814 A→C, $n = 3$; 1815 T→A, $n = 1$; 1816 G→T, $n = 1$; 1816 G→A, $n = 1$). Strains from two individuals had double mutations at codons 1 and 28 (1814 A→C and 1896 G→A; 1816 G→T and 1896 G→A). Twelve HBeAg-negative, anti-HBe-positive carriers were highly viremic ($\geq 10^5$ HBV-DNA copies/ml serum), with 8 of them infected with a precore mutant strain.

Of the additional 26 HBeAg-negative, anti-HBe-positive patients in group B, 13 carried a precore mutant. Ten mutations introduced a stop in codon 28 (1896 G→A, $n = 8$; 1897 G→A, $n = 2$), and 3 abolished the initiation codon (1814 A→T, $n = 2$; 1814, A→C, $n = 1$).

An additional sequence change 1899 G→A (changing glycin to aspartic acid at codon 29) was found in 22 of 32 individuals with the 1896 G→A stop mutation. In 18 of these 22 carriers wild-type sequence 1899 G was present as well. This 1899 G→A variant was also found, in the presence of wild-type sequence 1899 G, in 11 of 67 individuals without a stop mutation at position 1896.

Comparison of HBeAg-Negative, Anti-HBe-Positive Virus Carriers With and Without Precore Mutants

Carriers of wild-type and carriers of precore mutant HBV among the HBeAg-negative, anti-HBe-positive virus carriers of group A were compared regarding age, sex, level of viremia, alanine transaminase (ALT) level, aspartate transaminase (AST) level, chronic sequelae of infection (hepatic cirrhosis and/or hepatocellular carcinoma), and, where available, travel history and duration of disease. Statistically significant differences were not found (Table III).

Group B patients had ALT elevations (61% vs. 38%) more often, and a higher incidence of clinical sequelae (35% vs. 16%) than individuals from group A. Five patients of this group, but none of group A, had been treated with interferon. Nevertheless, as in group A, no significant differences were found in group B between carriers of precore wild-type and those of mutant strains with the parameters mentioned above as well as interferon treatment.

When searching simultaneously for all associations between the presence of a mutation, clinical sequelae, and age (age groups <40, 40–59, and ≥60 years), no association was found between the presence of a precore mutation and age or sequelae. The 1899 G→A sequence variant had no effect on the clinical condition or level of viremia in any group.

All mutations leading to a stop in codon 28 ($n = 32$) and all mutations abolishing the start codon ($n = 11$) were combined, to determine whether the type of mutation was associated with a particular profile of disease. No differences were found between carriers of wild-type, precore start, and precore stop mutations in relation to the parameters listed in Table III, by univariate analysis for patient groups A and B alone and combined.

DISCUSSION

More than half (44/84) of the anti-HBe-positive HBV carriers of German origin were found to be infected with a precore mutant strain. This number is even higher than the percentage described previously by Tillmann et al. [1995], who studied 30 unselected anti-HBe-positive German virus carriers, 12 of whom had precore mutants. The only other German study published so far [Dienes et al., 1995] was restricted to anti-HBe-positive patients with severe chronic active hepa-

TABLE III. Comparison of 81 HBeAg-Negative, Anti-HBe-Positive German Individuals Chronically Infected With Either HBV Wildtype or Precore Mutant Strains[a]

	Group A (n = 58)		Group B (n = 23)		Group A + B (n = 81)	
	Wildtype (n = 27)	Mutant (n = 31)	Wildtype (n = 12)	Mutant (n = 11)	Wildtype (n = 39)	Mutant (n = 42)
Age (years)[b]	49.3 ± 15.3	50.9 ± 15.6	41.2 ± 15.1	49.4 ± 19.1	46.8 ± 15.5	50.5 ± 16.4
Number of males	22 (81%)	23 (74%)	10 (83%)	11 (100%)	32 (82%)	34 (81%)
Patients with $\geq 10^5$ HBV-DNA copies/ml serum[d]	4 (15%)	8 (26%)	3 (25%)	3 (27%)	7 (18%)	11 (26%)
ALT levels (U/l)[c,e]	17 (9–243)	14 (4–222)	27 (10–62)	26 (6–338)	21 (9–243)	16 (4–338)
AST levels (U/l)[c,e]	13 (7–89)	11 (6–222)	22 (8–98)	19 (7–157)	19 (7–98)	12 (6–222)
Patients with cirrhosis/carcinoma[d]	5 (19%)	4 (13%)	4 (33%)	4 (36%)	9 (23%)	8 (19%)
Mean duration of disease (year)[b,f] (n)[g]	11.8 ± 6.3 (12)	14.8 ± 12.4 (12)	10.7 ± 9.4 (7)	10.4 ± 9.2 (7)	11.4 ± 7.6 (19)	13.2 ± 11.5 (19)

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine transaminase; AST, aspartate transaminase.
[a]Included were those 81 individuals for whom clinical data could be obtained.
[b]Mean ± SD.
[c]Median (range).
[d–f]No significant differences between HBV wildtype and precore mutant carriers when tested by [d]Fisher's exact test, [e]Mann–Whitney U test, or [f]t-test ($P \geq .11$ in all tests).
[g]Number of patients for whom information about minimal or exact duration of disease was available.

titis B: 22 of the 32 patients carried precore mutants, but the ethnic origin of these patients was not mentioned.

Although a variety of mutations prevent HBeAg synthesis [Akarca et al., 1994; Lok et al., 1994], many clinical studies were limited to screening for the predominant 1896 G→A stop mutation alone. In the present study, this approach would have missed 33% (16/48) of all identified precore mutations, thus, sequencing the complete precore region seems important for the identification of all possible mutations. Using this approach, a rare stop mutation 1897 G→A that was always accompanied by a C→T substitution at position 1857 (changing proline to leucine at codon 15), was found in four patients. This observation supports the proposal of an assumed best base pairing in the stem loop structure of the pregenomic HBV-RNA [Lok et al., 1994]. Mutations that abolished the precore translation initiation codon were found in 14% (12/84) of the anti-HBe-positive virus carriers.

About half of the HBeAg negative carriers had wildtype sequence throughout the entire precore region. One can only speculate why these HBV-DNA-positive carriers did not express HBeAg. One possible cause could be a mutation in the promotor or other regulatory regions influencing the expression of HBeAg. Further studies will be necessary to investigate this hypothesis.

There is still some confusion about the impact of precore mutants on the course of chronic HBV infection. The 1896 G→A mutation has been reported in association with flare-ups of liver cell necrosis [Brunetto et al., 1991], elevated ALT levels [Lopez Alcorocho et al., 1994], and longer duration of hepatitis [Mangia et al., 1996]. Other investigators, however, have found that the appearance of precore mutants is not associated with reactivation of chronic active hepatitis [Loriot et al., 1995], and that the presence of the wild-type precore sequence is associated with more severe fibrosis and inflammation [Lindh et al., 1996]. However, these studies are hardly comparable as they included different numbers of patients, patients from different ethnic origins, and a different spectrum of mutations. The present investigation focused on all mutations with proven functional significance for HBeAg production and included by far the largest number of anti-HBe-positive carriers with a homogeneous ethnic and geographic origin. For the 81 chronic carriers for whom clinical data could be obtained, no differences were found between carriers of wild-type and precore mutant HBV with respect to age, sex, viremia, ALT and AST levels [ter Borg et al., 1998], clinical status, previous interferon therapy, and travel history. Furthermore, there was no difference between carriers with the 1896 G→A mutation and carriers with mutations elsewhere.

The results of this study do not confirm the findings on an adverse [Brunetto et al., 1991; Lopez Alcorocho et al., 1994] or beneficial [Lindh et al., 1996] influence of a precore mutation on chronic HBV infection. Tillmann et al. [1995] reported a more severe clinical course only in those six patients in whom a double mutation 1896 G→A and 1899 G→A was present, but not in patients with the 1896 G→A mutation alone. In the present study, 20 carriers with this double mutation were identified: they did not show a different clinical picture than the 8 carriers of the 1896 G→A mutation alone.

A lack of association between the presence of precore mutants and the clinical course can also be assumed due to the fact that there was no difference in the prevalence of precore mutants between an unselected population (group A), and a preselected patient group with a more severe clinical course (group B). Furthermore, antiviral therapy with interferon does not seem to have promoted the emergence of HBV variants in patient group B.

In conclusion, the present study confirms that precore mutants are frequent in native Germans. This important finding concerns the interpretation of serologi-

cal results, since anti-HBe-positive carriers are thought usually to have no or only small numbers of circulating virions, but carriers of these mutants sometimes have significant viremia. However, there seems to be no association between the presence or the location of a precore mutation and increased severity of chronic HBV infection.

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